Number of Circulating Endothelial Progenitor Cells and Intratumoral Microvessel Density in Non-small Cell Lung Cancer Patients

Differences in Angiogenic Status between Adenocarcinoma Histologic Subtypes

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Introduction: Angiogenesis plays a significant role in tumor progression. This study examined the association between the number of circulating endothelial progenitor cells (EPCs), intratumoral microvessel density (MVD) (both of which may be markers for neovascularization), and lung cancer histological types, particularly adenocarcinoma histological subtypes.

Methods: A total of 83 stage I non-small cell lung cancer (NSCLC) patients underwent complete tumor resection between November 2009 and July 2010. The number of EPCs from the pulmonary artery of the resected lungs was measured by assaying $CD34^+$/vascular endothelial growth factor receptor 2 positive cells, and the MVD was assessed immunohistochemically in tumor specimens by staining for CD34.

Results: A statistically significant correlation between the number of EPCs from pulmonary artery and intratumoral MVD was found ($p < 0.001$). No statistically significant differences in the number of EPCs and the MVD were observed between the adenocarcinomas and the squamous cell carcinomas. Among the adenocarcinoma histological subtypes, a higher number of EPCs and MVD were found significantly more frequently in solid adenocarcinomas than in nonsolid adenocarcinomas ($p < 0.001$ and $p = 0.011$, respectively). In addition, solid adenocarcinomas showed higher levels of vascular endothelial growth factor using quantitative real-time polymerase chain reaction in the tumor tissue samples than in the nonsolid adenocarcinomas ($p = 0.005$).

Conclusion: The higher number of circulating EPCs and the MVD of solid adenocarcinoma may indicate the presence of differences in the tumor angiogenic status between early-stage adenocarcinoma histological subtypes. Among adenocarcinoma patients, patients with solid adenocarcinoma may be the best candidates for antiangiogenic therapies.

Key Words: Non-small cell lung cancer, Adenocarcinoma, Subtypes, Angiogenesis, Circulating endothelial progenitor cell.

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Lung cancer is the leading cause of death from cancer in the world,[1] and non-small cell lung cancer (NSCLC) accounts for the majority of lung cancers.[2] Among NSCLC histological types, adenocarcinoma of the lung is the most frequent histological type, and its incidence is increasing in most countries.[3] In Japan, adenocarcinoma is the most common histological type of resected lung cancer, accounting for more than 60% of all cases.[4]

Adenocarcinomas are categorized into four histological subtypes based on morphology: bronchioloalveolar carcinoma (BAC), acinar, papillary, and solid adenocarcinoma.[5] Among these major histological subtypes of lung adenocarcinoma, BAC is often reported to be associated with a favorable prognosis,[6–8] whereas the other subtypes are considered invasive and are associated with unfavorable outcomes.[9,10]

Treatment of lung adenocarcinoma is now moving beyond conventional chemotherapy, with the advent of molecular-targeted therapies such as epidermal growth factor receptor (EGFR) inhibitors.[11] An EGFR mutation was found specifically in the BAC subtype, and differences in the frequency of EGFR mutations exist among the adenocarcinoma histological subtypes.[11]

Angiogenesis is essential for cancer growth and progression.[12] Another key feature of molecular-targeted therapies against lung adenocarcinoma is the inhibition of specific

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cytokines essential for tumor vascularization.[13] Although bevacizumab (Avastin; Genentech, South San Francisco, CA), a monoclonal antibody with an antiangiogenic effect that specifically antagonizes and blocks vascular endothelial growth factor (VEGF), had been shown to increase the overall survival of patients with advanced adenocarcinoma when administered in combination with standard chemotherapy,[13] which adenocarcinoma subtypes are most dependent on angiogenesis for their growth remains unclear.

Recent evidence shows that the tumor vasculature can arise through vasculogenesis, a process by which bone marrow–derived $CD34^+$/vascular endothelial growth factor receptor 2 $(VEGFR\text{-}2)^+$ endothelial progenitor cells (EPCs) are recruited and differentiated in situ into mature endothelial cells to form new blood vessels.[14,15] The level of circulating EPCs has been reported to be a potential marker for neovascularization and for the response to antiangiogenic therapies.[16] Other studies have also reported a significant correlation between neovascularization, as assessed by the intratumoral microvessel density (MVD), and patient outcome in a variety of tumors.[17–19]

The addition of antiangiogenic therapy to conventional chemotherapy has recently become a standard therapy for lung adenocarcinoma.[13] However, the exact relationship between specific adenocarcinoma subtypes and the number of EPCs or MVD, both of which may be markers for neovascularization or the response to antiangiogenic therapies, has not been addressed thus far. The evaluation of these relationships could lead to the development of a relatively simple pathological examination of lung adenocarcinoma to determine a patient's sensitivity to antiangiogenic-targeted treatment and may help to identify the biological differences between adenocarcinoma histological subtypes. This study investigated the association between the number of circulating EPCs, MVD, and lung cancer histological types, with particular emphasis on adenocarcinoma subtypes.

PATIENTS AND METHODS

Patients

A total of 122 consecutive Japanese patients with stage I NSCLC underwent complete tumor resection with a lobectomy or a more extensive surgery between November 2009 and July 2010 at the National Cancer Center Hospital East. Among these patients, 39 were excluded because of (1) preoperative chemotherapy and/or radiation therapy ($n = 2$) or (2) unavailability of blood samples from the resected lungs ($n = 37$). The remaining 83 patients were included in this study.

Pathological Evaluation

All surgical specimens were fixed with 10% formalin and embedded in paraffin wax. The tumors were cut at approximately 5-mm intervals, and serial 4-μm sections were stained with hematoxylin-eosin. The disease stages were diagnosed based on the tumor, node, and metastasis (TNM) classification of the International Union Against Cancer, 7th edition.[20] The histological type was determined according to the classification of the World Health Organization.[5] We diagnosed squamous cell carcinoma based on the findings of keratinization, intercellular bridges, and squamous pearl formation. Adenocarcinoma histological subtypes were categorized into BAC (nonmucinous or mucinous), papillary, acinar, and solid adenocarcinomas according to the classification of the World Health Organization.[5] Mucin production in a solid adenocarcinoma component was confirmed using the Alcian blue-periodic acid Schiff method. All the adenocarcinomas were divided according to the predominant subtype into four subgroups: BAC, papillary, acinar, and solid adenocarcinoma with mucin production. Tumor size was measured as the maximal diameter of the tumor on the cut sections. Intratumoral vascular invasion and visceral pleural invasion were evaluated using staining with hematoxylin-eosin and Victoria blue-van Gieson stains.

Blood Sampling from the Pulmonary Artery of Resected Specimens

Human monocytes were isolated from the pulmonary artery (PA) of resected lungs as previously reported.[21,22] In brief, the dissected and ligated PA of surgically resected lungs contains more than 4 ml of blood. In this study, a 21-gauge needle was inserted into the PA of 83 lungs surgically resected from stage I primary NSCLC patients at our hospital. All the specimens were collected after the patients had given their written informed consent, and the study was approved by the Institutional Review Board of the National Cancer Center.

Flow Cytometry

Blood samples from the PA of the resected lungs were processed within 1 hour after collection. Blood mononuclear cells from the PA were prepared by gradient centrifugation using Ficoll-Hypaque. The expression of cell surface antigens was determined using two-color immunofluorescence staining. In brief, 100 μl of blood mononuclear cells (containing 5×10^5 cells) were incubated with 40 μl of FcR-blocking reagent (MBL, Aichi, Japan) for 10 minutes to inhibit nonspecific bindings. Subsequently, the cells were incubated at 4°C for 15 minutes with 10 μl of phycoerythrin-conjugated antihuman CD34 mAb (BioLegend, Bergisch-Gladbach, Germany) and 20 μl of allophycocyanin-conjugated VEGFR-2 mAb (R&D Systems, Wiesbaden-Nordenstadt, Germany). Phycoerythrin (PE)- and allophycocyanin (APC)-conjugated isotype-matched immunoglobulin Ig-G1 (Abcam, Cambridge, United Kingdom) and Ig-G2a (DakoCytomation, Hamburg, Germany) antibodies were used as negative controls. The cells were washed three times to remove unbound antibodies and finally resuspended in 500 μl of fluorescence activated cell sorting (FACS) solution. A FACS analysis was performed using a FACSCalibur flow cytometer (BD Bioscience, Heidelberg, Germany). A minimum of 10,000 events were collected.

Immunohistochemistry

After reviewing the hematoxylin-eosin-stained slides of the surgical specimens, the block containing the most extensive tumor component was selected from each specimen. Sections (4 μm each) were cut from the paraffin blocks and

mounted on silanized slides. The sections were deparaffinized in xylene, dehydrated in a graded ethanol series, washed with distilled water, and placed in 0.1 M citric acid buffer. For antigen retrieval, the slides were heated at 95°C for 20 minutes in a microwave oven and then allowed to cool for 1 hour at room temperature. Next, the slides were washed three times in phosphate-buffered saline (PBS) and immersed in a 0.3% hydrogen peroxide solution in methanol for 15 minutes to inhibit endogenous peroxidase activity. After washing the slides three times in PBS, nonspecific binding was blocked by preincubation with 2% normal swine serum in PBS (blocking buffer) for 30 minutes at room temperature. Individual slides were then incubated overnight at 4°C with mouse anti-CD34 antibody (R&D Systems) at a final dilution of 1:50 in blocking buffer. The slides were again washed three times with PBS, incubated with EnVision (DAKO, Tokyo, Japan) for 1 hour at room temperature, and after extensive washing with PBS, the color reaction was developed for 2 minutes in 2% 3,3′-diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen peroxide. Finally, the sections were counterstained with Meyer's hematoxylin, dehydrated, and mounted. The three most vascular areas (hot spots) in the invasive foci within a section were selected for the quantification of angiogenesis, and vessels labeled with the anti-CD34 mAb were counted under light microscopy at a magnification of ×400 (×400; ×40 objective, and ×10 ocular; 0.196 mm^2/field) based on previous reports.[18–23] Each single or connected endothelial cell that stained in these areas was counted as a microvessel. The average counts were recorded as the CD34-MVD for each case.

Tissue Samples, RNA Extraction, Reverse Transcription, and Real-Time Polymerase Chain Reaction

Total RNA was extracted from 27 adenocarcinoma patients who had undergone surgical resection at our hospital. Samples of cancer tissue were collected and immediately homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) with Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and stored at −80°C until use. Total RNA was isolated from the tissues using a commercial RNA isolation reagent according to the manufacturer's instructions. The RNA was reverse transcribed to synthesize cDNA using a PrimerScript RT reagent kit according to the manufacturer's instructions (Takara Biochemicals, Shiga, Japan).

To quantitatively compare the mRNA level of VEGF-A, RT-PCR was performed in a Smart Cycler System (TaKaTa) using SYBR Premix Ex Taq (TaKaRa). The sense and antisense primers used for the quantitative amplification of VEGF mRNAs were 5′-GAGCCTTGCCTTGCTGCTCTAC-3′ and 5′-CACCAGGGTCTCGATTGGATG-3′, and the primers used for the amplification of glyceraldehyde-3-phosphate dehydrogenase as an internal control were 5′-GCACCGTCAAGGCTGAGAAC-3′ and 5′-ATGGTGGTGAAGACGCCAGT-3′.

The amount of template cDNA was expressed by a threshold cycle (G) that was determined from the amplification curve (exponential curve) and a threshold level of PCR product detection. One G was equal to a twofold difference in the initial template. The quantification data were analyzed using Smart Cycler System software, version 2.0d (Cepheid). The level of VEGF expression was reported as the ratio of its expression to the level of *GAPDH* gene expression in the same sample.

Clinicopathological Data

The medical records of all the patients were reviewed to obtain the clinicopathological data, which included age (dichotomized according to a median age of 69 years), sex, smoking history (nonsmokers or ever-smokers), diameter of the tumor on the resected specimens (dichotomized according to a diameter of 3.0 cm), histological type (adenocarcinoma, squamous cell carcinoma, or others), lymphatic permeation (present or absent), intratumoral vascular invasion (present or absent), and visceral pleural invasion (as defined in the TNM classification, 7th edition[17]; present or absent).

Data and Statistical Analysis

All data are presented as the mean ± SE. Differences in categorical outcomes were evaluated using the χ^2 test. Continuous variables were compared using *t* tests. All the reported *p* values were two-sided, and the significance level

TABLE 1. Patient Characteristics

Characteristics	No. of Patients (%)
Total	83 (100)
Age (yr)	
<69	41 (49)
≥69	42 (51)
Sex	
Women	30 (36)
Men	53 (64)
Smoking history	
Never-smoker	32 (39)
Ever-smoker	51 (61)
Tumor size (cm)	
≤3	62 (75)
>3	21 (25)
Histologic type	
Adenocarcinoma	63 (76)
Predominant subtype	
BAC	23
Papillary	12
Acinar	18
Solid	10
Squamous cell carcinoma	15 (18)
Others	5 (6)
Lymphatic permeation	
Absent	77 (93)
Present	6 (7)
Intratumoral vascular invasion	
Absent	59 (71)
Present	24 (29)
Visceral pleural invasion	
Absent	69 (83)
Present	14 (17)

BAC, bronchioloalveolar carcinoma.

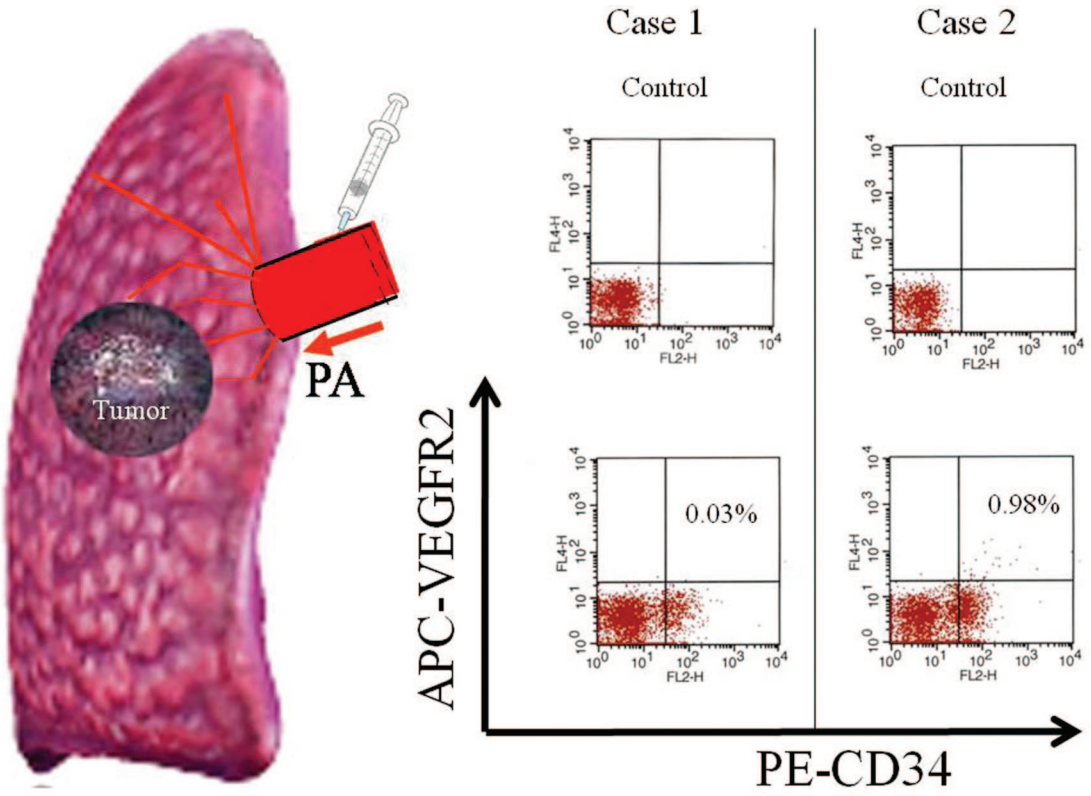

FIGURE 1. Quantification of circulating endothelial progenitor cells (EPCs) in blood mononuclear cells from the pulmonary fraction. Representative data from two flow cytometry analyses are shown. PA, pulmonary artery; VEGFR2, vascular endothelial growth factor receptor 2; APC, allophycocyanin; PE, phycoerythrin.

was set at less than 0.05. Analyses were performed using SPSS version 11.0 (Dr. SPSS II for Windows, standard version 11.0; SPSS Inc., Chicago, IL).

RESULTS

Patient Characteristics

This study analyzed 83 patients with completely resected stage I NSCLC, including 53 men and 30 women with a median (range) age of 69 years (42–82 years). Thirty-two patients were never-smokers, and 51 were ever-smokers. The patient characteristics are shown in Table 1.

Sixty-three patients had adenocarcinoma, 15 had squamous cell carcinoma, and 5 had other histological subtypes. The 63 patients with adenocarcinoma were classified according to the predominant subtypes as follows: 23 patients had BAC, and 12 patients had papillary, 18 patients had acinar, and 10 patients had solid adenocarcinomas. Of the 83 patients included in this study, 21, 6, 24, and 14 patients had tumors with a large tumor size (≥3 cm), lymphatic permeation, intratumoral vascular invasion, and pleural invasion, respectively (Table 1).

EPCs from PA in Stage I NSCLC and Correlation between Clinicopathological Characteristics and the Number of EPCs

Figure 1 shows the EPC quantification in blood mononuclear cells from the PA fraction and representative data for two flow cytometry analyses. Table 2 shows the number of EPCs from the PA fraction according to the clinicopathological features in all the patients. No statistically significant correlations were observed between the number of EPCs and the patient's age, sex, smoking history, tumor size, histological types, lymphatic permeation, vascular invasion, or visceral pleural invasion (Table 2).

MVD in Stage I NSCLC and Correlation between Clinicopathological Characteristics and MVD

The median microvessel count for all patients was 16.2 (per 400× field), with an interquartile range of 0 to 78.7. Representative tissue specimens from stage I NSCLC patients are shown in Figure 2 for adenocarcinomas with a low MVD (Figure *2A*) and a high MVD (Figure *2B*) and squamous cell carcinomas with a low MVD (Figure *2C*) and a high MVD (Figure *2D*). Patients were classified into two groups with a high MVD (>16.2) or a low MVD (≤16.2) based on the median MVD of the entire group.

Table 3 lists the MVD in correlation with the clinicopathological features in all the patients. No statistically significant correlations were observed between the MVD and the patient's age, sex, smoking history, tumor size, histological type, lymphatic permeation, vascular invasion, or visceral pleural invasion. In contrast, a statistically significant corre-

TABLE 2. Correlation between Clinicopathological Characteristics and the Number of EPCs in the Entire Cohort

Characteristics	No. of Patients (%)	No. of EPC from PA (/ml) ± SE	*p*
Total	83 (100)	2619 ± 372	
Age (yr)			
<69	41 (49)	2829 ± 569	0.580
≥69	42 (51)	2414 ± 486	
Sex			
Women	30 (36)	1730 ± 315	0.072
Men	53 (64)	3122 ± 546	
Smoking history			
Never-smoker	32 (39)	1768 ± 342	0.069
Ever-smoker	51 (61)	3153 ± 556	
Tumor size (cm)			
≤3	62 (75)	2456 ± 409	0.454
>3	21 (25)	3100 ± 846	
Histologic type			
Adenocarcinoma	63 (76)	2420 ± 397	
Squamous cell carcinoma	15 (18)	3527 ± 1204	0.272[a]
Others	5 (6)	2403 ± 429	0.991[a]
Lymphatic permeation			
Absent	77 (93)	2459 ± 358	0.123
Present	6 (7)	4678 ± 2345	
Intratumoral vascular invasion			
Absent	59 (71)	2193 ± 356	0.072
Present	24 (29)	3667 ± 923	
Visceral pleural invasion			
Absent	69 (83)	2480 ± 389	0.409
Present	14 (17)	3306 ± 1105	

[a] Compared with adenocarcinoma.
EPC, circulating endothelial progenitor cell; PA, pulmonary artery.

lation was observed only between the MVD and the number of EPCs ($p < 0.001$). In the high MVD group, the number of EPCs was significantly higher than that in the low MVD group (mean = 4380 ± 620 and 901 ± 185, respectively; Figure 3).

Correlation between the Number of EPCs and Adenocarcinoma Histological Subtypes

Figure 4*A* shows the number of EPCs in stage I adenocarcinoma patients stratified according to their predominant histological subtypes. The number of EPCs in patients with predominantly solid adenocarcinomas (mean = 5776 ± 1720/ml) was significantly higher than that among patients with predominantly BAC (mean = 1643 ± 420/ml, $p = 0.003$), papillary adenocarcinoma (mean = 2140 ± 770/ml, $p = 0.048$), or acinar adenocarcinoma (mean = 1734 ± 394/ml, $p = 0.007$).

No statistically significant differences in the number of EPCs were observed among patients with predominantly BAC, papillary adenocarcinoma, and acinar adenocarcinoma. Therefore, patients with predominantly BAC, papillary adenocarcinoma, or acinar adenocarcinoma were grouped as nonsolid adenocarcinomas patients and were compared with patients with predominantly solid adenocarcinomas in the following analyses (Figure *4B*).

Differences in MVD and VEGF Levels in Tumor Tissue Samples between Solid and Nonsolid Adenocarcinoma Patients as Determined Using Quantitative Real-Time PCR

Table 4 shows the differences in the MVD between solid and nonsolid adenocarcinoma patients. The high-MVD group included a significantly higher number of solid adenocarcinoma patients than those with nonsolid adenocarcinoma patients ($p = 0.011$).

Because VEGF-A has been reported to play a crucial role in the recruitment of EPCs[24,25] and angiogenesis can be assessed according to the MVD,[26,27] the level of mVEGF-A

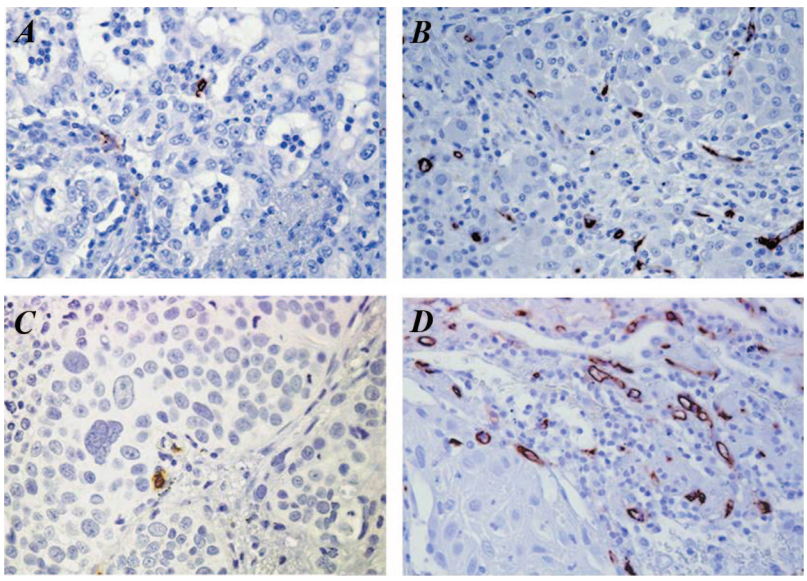

FIGURE 2. Immunohistochemical staining of non-small cell lung cancer (NSCLC) tissue with anti-CD34 antibodies for the quantification of microvessel density (MVD). Sections from specimens of adenocarcinoma with a low MVD (*A*) and a high MVD (*B*) and squamous cell carcinoma with a low MVD (*C*) and a high MVD (*D*) are shown. Original magnification, ×400. EPC, endothelial progenitor cell; PA, pulmonary artery.

TABLE 3. Correlation between Clinicopathological Characteristics and the Number of EPCs and MVD in the Entire Cohort

Characteristics	No. of Patients (%)	MVD		p
		Low[a]	High[b]	
Total	83 (100)	42	41	
Age (yr)				
<69	41 (49)	18 (44)	23 (56)	0.228
≥69	42 (51)	24 (57)	18 (43)	
Sex				
Women	30 (36)	17 (57)	13 (43)	0.406
Men	53 (64)	25 (47)	28 (53)	
Smoking history				
Never-smoker	32 (39)	19 (59)	13 (41)	0.205
Ever-smoker	51 (61)	23 (45)	28 (55)	
Tumor size (cm)				
≤3	62 (75)	32 (52)	30 (48)	0.752
>3	21 (25)	10 (48)	11 (52)	
Histologic type				
Adenocarcinoma	63 (76)	36 (57)	27 (43)	0.093
Squamous cell carcinoma	15 (18)	5 (33)	10 (67)	
Others	5 (6)	1 (20)	4 (80)	
Lymphatic permeation				
Absent	77 (93)	40 (52)	37 (48)	0.38
Present	6 (7)	2 (33)	4 (67)	
Intratumoral vascular invasion				
Absent	59 (71)	33 (56)	26 (44)	0.128
Present	24 (29)	9 (38)	15 (62)	
Visceral pleural invasion				
Absent	69 (83)	37 (54)	32 (46)	0.222
Present	14 (17)	5 (36)	9 (64)	
No. of EPC from PA/ml ± SE	83 (100)	901 ± 185	4380 ± 620	<0.001[c]

Numbers in parentheses are in percentages.
[a] ≤median.
[b] >median.
[c] Significance.
MVD, microvessel density; EPC, circulating endothelial progenitor cell; PA, pulmonary artery.

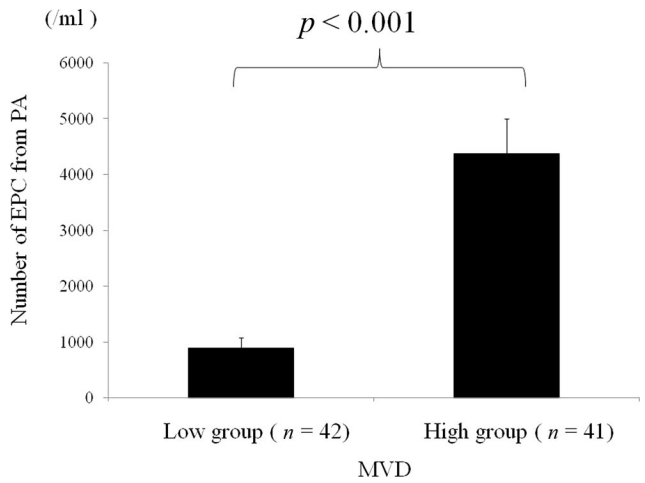

FIGURE 3. Correlation between the microvessel density (MVD) and the number of endothelial progenitor cells (EPCs) in the entire cohort. PA, pulmonary artery.

was measured using quantitative real-time PCR in the tumor tissue samples and was compared between solid and nonsolid adenocarcinoma patients. The level of mVEGF-A was significantly higher among solid adenocarcinomas than among nonsolid adenocarcinomas (Figure 5, $p = 0.005$).

DISCUSSION

In the most recently reported series, adenocarcinoma was found to be the most common type of early-stage lung cancer.[3,4] The recent increase in the detection of early-stage adenocarcinoma in Japan can be attributed to a nationwide mass screening system.[28] The major histological subtypes of adenocarcinoma are characterized as BAC, acinar, papillary, and solid components. Several reports have described differences in survival between adenocarcinoma subtypes. BAC has specific radiological features and is reported to be significantly correlated with a favorable prognosis.[6–8] In contrast, patients with solid adenocarcinoma have significantly poorer outcomes than those with other histological subtypes, and solid adenocarcinoma is

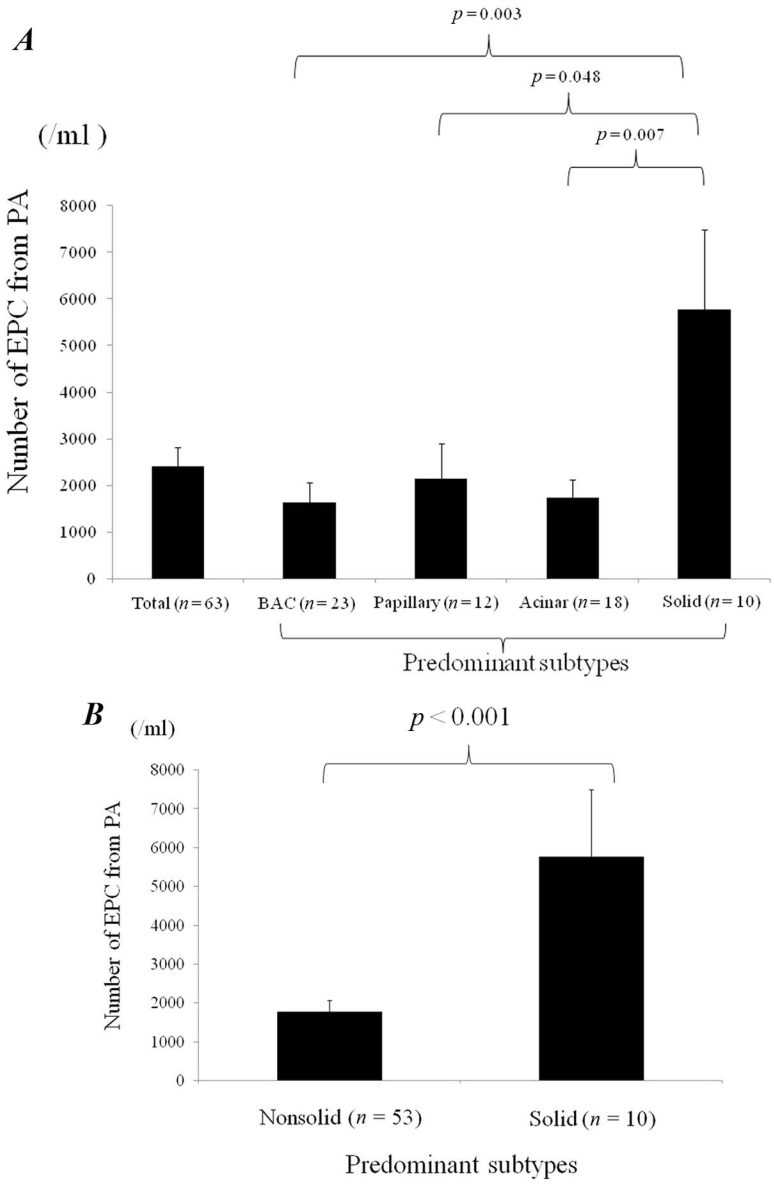

FIGURE 4. *A,* Correlation between the number of endothelial progenitor cells (EPCs) and adenocarcinoma histological subtypes. *B,* Differences in number of endothelial progenitor cells (EPCs) between solid and nonsolid adenocarcinoma patients. PA, pulmonary artery; BAC, bronchioloalveolar carcinoma.

TABLE 4. Differences in MVD between Solid and Nonsolid Adenocarcinoma Patients

Predominant Adenocarcinoma Subtypes	No. of Patients (%)	MVD		*p*
		Low[a]	High[b]	
Total	63	36 (57)	27 (43)	
Nonsolid subtypes	53 (84)	34 (64)	19 (36)	
Solid subtype	10 (16)	2 (20)	8 (80)	0.011[c]

Numbers in parentheses are in percentages.
[a] ≤median in the entire cohort.
[b] >median in the entire cohort.
[c] Significance.
MVD, microvessel density.

the most poorly differentiated among lung adenocarcinoma subtypes.[9,10] In addition, Ding et al.[11] reported that an EGFR mutation showed a significant positive correlation with BAC and papillary subtypes but not with the solid subtype. Thus, with the introduction of computed tomography screening and the discovery of targeted small molecule therapies against EGFR, there has been an enormous interest in the pathology, radiology, molecular biology, and clinical features of lung adenocarcinoma subtypes.

Several reports have revealed that angiogenesis plays a significant role in the pathogenesis of tumors and in the mechanisms of disease progression.[12,18] Recent reports have indicated that the transplantation of ex vivo cultivated EPCs reportedly contributes to angiogenic tumor vasculature.[29–31] Despite certain discrepancies in the existing reports,[32–34] the role of EPCs in vessel formation in tumors has now become widely accepted. Therefore, recent evidence demonstrating

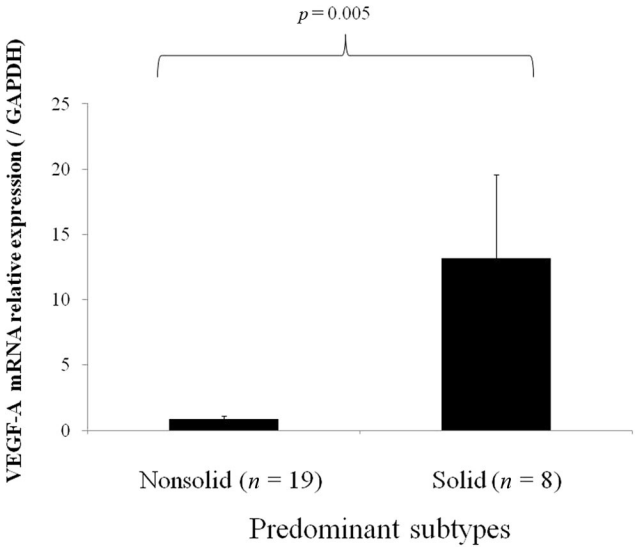

FIGURE 5. Differences in vascular endothelial growth factor (VEGF)-A levels in tumor tissue samples between solid and nonsolid adenocarcinoma patients, as determined using a quantitative real-time polymerase chain reaction. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the existence of a bone marrow reservoir of EPCs and their selective involvement in neovascularization has attracted considerable interest because these cells could be used as surrogate markers to monitor the status of tumor angiogenesis.[16] In this study, the number of EPCs in patients with predominantly solid adenocarcinoma was significantly higher than in patients with predominantly BAC, papillary, or acinar adenocarcinomas. The higher number of circulating EPCs associated with solid adenocarcinoma may indicate the presence of differences in the tumor angiogenic status between early-stage adenocarcinoma histological subtypes.

In addition to the number of circulating EPCs, several reports have also demonstrated a significant correlation between neovascularization assessed using the intratumoral MVD, the tumor angiogenic status, and patient outcome in a variety of tumors.[17–19] In this study, the MVD of the tumor was significantly correlated with the number of circulating EPCs. Regarding the prognostic relevance of angiogenic activity in NSCLC as expressed by the intratumoral MVD, a high MVD has been identified as an unfavorable prognostic factor.[35,36] Yuan et al.[37] reported that the MVD was significantly correlated with the histological types and that a higher MVD was found significantly more frequently in adenocarcinoma than in squamous cell carcinoma, suggesting that adenocarcinomas might have a higher angiogenic potential. However, in this study, no statistically significant differences in the MVD were observed between adenocarcinoma and squamous cell carcinoma. Instead, we found significant differences in the MVD among the adenocarcinoma histological subtypes. A higher MVD was found significantly more frequently in solid adenocarcinomas than in nonsolid adenocarcinomas. This may reflect the aggressive and invasive characteristics of this subtype and may be one of the reasons why patients with solid adenocarcinoma have significantly poorer outcomes than those with other adenocarcinoma histological subtypes.

VEGF is the most important angiogenesis factor, and its expression within tumors is suggested to affect the prognosis of patients.[26,38] Thus far, there have been several reports regarding the association between the level of VEGF-A and MVD.[26,27,36] In addition, bone marrow-derived EPCs are also reported to be mobilized by the stimulation of tumor-derived VEGF-A, inducing them to migrate toward the tumor and to become incorporated into the developing neovasculature.[24,25] In this study, we confirmed that higher levels of VEGF-A are present in solid adenocarcinomas than in nonsolid adenocarcinomas. Recent studies have shown that the addition of antiangiogenic therapy, such as bevacizumab, to paclitaxel and carboplatin improves survival, compared with chemotherapy alone, in patients with previously untreated metastatic nonsquamous NSCLC.[13] Especially among adenocarcinoma patients, those with a solid adenocarcinoma may be the best candidates for the addition of bevacizumab, an antiangiogenic monoclonal antibody that blocks VEGF-A.

In this study, we showed a difference in the number of circulating EPCs or intratumoral MVD, both of which might be potential markers for neovascularization, between adenocarcinoma histological subtypes. Gao et al.[39] reported that circulating EPCs play a major and catalytic role in tumor progression, which may be maximized in metastatic and relapsing disease by the promotion of the progression of avascular micrometastases to vascularized macrometastases. The significantly higher levels of EPCs paralleling clinical severity also suggest the possible relevance of these cells in the metastatic progression of the tumors[16] and point to their potential use as targets in therapy against metastatic sites. Therefore, preoperative or postoperative anti-EPC therapy may be indicated for early-stage adenocarcinoma patients with preoperative high EPC levels to prevent postoperative recurrence after resection. In this study, the number of EPCs in patients with solid adenocarcinoma was significantly higher than that in nonsolid adenocarcinoma patients. This finding may indicate a subgroup of adenocarcinoma patients who may benefit from angiogenesis inhibitors targeted against EPCs.

This study had several limitations. In particular, the study lacked ethnic diversity, as all the patients were Japanese. Another limitation is that the blood mononuclear cells from the PA were isolated from the resected lungs and not directly from the patients preoperatively to avoid unnecessary invasiveness. However, we believe that the level of EPCs in the blood from the PA in the vicinity of the tumor more precisely reflects the effect of the tumors than the samples from the peripheral blood, as previously reported.[16,24] In this study, we first reported the differences in the number of circulating EPCs or MVD between lung adenocarcinoma subtypes. Further clinical studies are needed to confirm the beneficial effects of antiangiogenic therapy against VEGF or EPCs in solid adenocarcinoma patients.

CONCLUSIONS

The number of EPCs and the MVD in patients with predominantly solid adenocarcinomas were significantly higher than those in nonsolid adenocarcinoma patients. In particular, patients with solid adenocarcinoma may be the best candidates for antiangiogenic therapies against VEGF or EPCs among the various adenocarcinoma subtypes.

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